AD NUMBER

ADB265839

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Jun 2000. Other requests shall be referred to U.S. Army Medical Research and Material Command, 504 Scott St., Fort Detrick, MD 21702-5012.

AUTHORITY

U.S. Army Medical Research and Materiel Command and Fort Detrick ltr., dtd October 17, 2001.

AD_____

Award Number: DAMD17-97-1-7057

TITLE: Characterization of New Breast Tumor-Specific Antigens Using a Novel Antigen Discovery System

PRINCIPAL INVESTIGATOR: Henry Kao
Olivera J. Finn, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, Pennsylvania 15201

REPORT DATE: June 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-97-1-7057
Organization: University of Pittsburgh
Location of Limited Rights Data (Pages):

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

Patricia C. Modrow 4/19/01

_____ _____

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2000	Final (1 Jun 97 - 31 May 00)

4. TITLE AND SUBTITLE
Characterization of New Breast Tumor-Specific Antigens Using a Novel Antigen Discovery System

5. FUNDING NUMBERS
DAMD17-97-1-7057

6. AUTHOR(S)
Henry Kao
Olivera J. Finn, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pittsburgh
Pittsburgh, Pennsylvania 15201

E-MAIL:
hekst5@pitt.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Jun 00). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The goal of this project is to devise a new method for antigen discovery using dendritic cells as antigen-presenting cells to prime autologous naïve $CD4^+$ and $CD8^+$ T cells from healthy donors against breast tumor proteins and peptides. To identify HLA Class I-restricted tumor antigens, we extracted peptides from tumor HLA Class I molecules, fractionated them by RP-HPLC, and loaded them onto DCs to prime naïve $CD8^+$ T cells *in vitro*. Our results show that we were able to prime naïve $CD8^+$ T cells to several peptide fractions and generate specificity to the tumor. Electrospray ionization mass spectrometry of the immunostimulatory peptide fractions identified a panel of peptides that could bind MHC-Class I molecules. To identify HLA Class II-restricted tumor antigens, we fractionated tumor protein extracts using RP-HPLC, and loaded them onto DCs to prime naïve $CD4^+$ T cells *in vitro*. Our results show that we were able to prime naïve $CD4^+$ T cells to several protein fractions and generate specificity to the tumor. In summary, results stemming from this project show that we have created a novel method that identified a panel of potentially new MHC Class I- and Class II-restricted tumor antigens.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Predoc Award	47
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Deparment of Army endorsement or approval of the products or services of these organizations.

_X_ In conducting research using animals, the investigator (s) adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, revised 1985).

_X_ For the protection of human subjects, the investigator (s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator (s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator (s) adhered to the NIH guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator (s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Henry Kau 8/28/00

PI - Signature Date

Table of Contents

INTRODUCTION

The prospect of successful immunotherapy against breast cancer relies in part on the discovery of tumor-specific antigens and their ability to stimulate immune responses in the host. Various approaches have been developed to search for new tumor-specific antigens, i.e. the genetic approach (1, 2), the peptide elution approach (3, 4), the reverse immunology approach (5, 6), and SEREX (7). However, all these approaches depended on the availability of T cell lines or clones from cancer patients that could be used to screen new tumor targets. A panel of melanoma tumor antigens (8) have been identified using these approaches, but very few have been found in tumors of other types, such as breast tumors. Studies have also shown that T cells from cancer patients may be defective (9), thus providing another caveat in using these T cells effectively as reagents to identify new tumor antigens. The goal of this project is to alleviate these concerns and create a new tumor antigen discovery system to identify new breast tumor-specific antigens. We hypothesize that by using dendritic cells from healthy donors to prime naïve T cells to breast tumor proteins and peptides *in vitro*, we could circumvent the problem of tumor-induced immunosuppression *in vivo* and identify new breast tumor-specific antigens.

BODY

I. Technical Objectives

The project has followed the original technical objectives, with emphasis placed on Technical Objectives 1 & 2:

1. To use human dendritic cells to prime *in vitro* naïve, autologous $CD8^+$ T cells to peptides eluted from HLA Class I molecules of the tumor, MS
2. To use human dendritic cells to prime naïve, autologous $CD4^+$ T cells to fractionated tumor cell lysates of the tumor, MS
3. To clone the genes pertinent to the identified immunogenic tumor peptides and/or proteins

II. Studies & Results

Identification of HLA Class I-restricted tumor antigens (Relevant to Technical Objectives 1 & 3)

To focus on peptides eluted from HLA-A2.1, the most common allele from the Caucasian population, we transfected the MS tumor with the HLA-A2.1 allele (henceforth denoted as MS-A2), as described in the 1998 DOD Annual Report. To address **Task 1** of the *Statement of Work*, we generated large numbers of MS-A2 tumor cells in culture, purified the HLA Class I molecules by immunoaffinity chromatography, acid-extracted the HLA bound peptides, and fractionated them by RP-HPLC. Our results showed that we were able to purify HLA Class I molecules and acid-extract the peptides with reasonable success (10). Electrospray ionization tandem mass spectrometry analysis of these HPLC fractions showed a panel of HLA Class I-bound peptides (Table 1), demonstrating our success in extracting peptides bound to tumor HLA Class I molecules (10,11). Details of these methods have been described in the 1998 DOD

Annual Report as well as a manuscript that is in press for the journal *Clinical Cancer Research* (See Appendix).

To address **Task 2** of the *Statement of Work*, we loaded dendritic cells from an HLA-A2.1$^+$ healthy donor with individual HPLC peptide fractions to prime naïve, autologous CD8$^+$ T cells. Our results showed that we were able to prime CD8$^+$ T cells to these peptide fractions and generate specificity to the breast tumor from which the peptides were derived (Figure 1, ref. 11). Interestingly, one of the primed CD8$^+$ T cell cultures recognized both an HLA-A2.1$^+$ breast tumor, MS-A2, and lung tumor, 201T-A2, suggesting the presence of shared tumor antigens between these tumors (Figure 2, ref. 11). Most importantly, we were able to reproducibly prime naïve CD8$^+$ T cells to peptides collected from different HPLC runs (Figure 3, ref. 11) demonstrating our success in priming CD8$^+$ T cells to eluted peptides and in generating tumor-specific T cell lines. From this part of the work, we have identified 12 HPLC fractions that contain potential breast tumor antigen candidates (Table 1, ref. 11).

To determine the biochemical content and identity of the immunostimulatory HPLC fractions, we analyzed the peptide fractions by electrospray ionization mass spectrometry as proposed for **Task 7** of the *Statement of Work*. Our results showed that we have identified a panel of peptides that conform to mass-to-charge ratios of peptides that bind to HLA Class I molecules (Table 1, ref. 11). Our initial sequencing efforts by tandem mass spectrometry yielded a 7 amino acid peptide that bound to HLA-A3 (12), but subsequent experiments showed tht it did not confer immunostimulatory capacity. In collaboration with Dr. Donald Hunt from the University of Virginia, we have sequenced peptides from another fraction and found 6 interesting tumor peptides that all bound to HLA-A2.1 with various affinities (Table 2), all having the same sequence except at the C-terminus.

Table 2. HLA Binding Specificity of Peptides from Fraction #50

	HLA-A2[a]	HLA-A3[b]
Peptide #1	++[c]	-
Peptide #2	+	-
Peptide #3	+	-
Peptide #4	++	-
Peptide #5	++	-
Peptide #6	+++	-

[a] T2 cells were loaded with the indicated peptides for 18 hours at 27^{0}C, and stained for HLA-A2.1 expression using the MA2.1 antibody.

[b] Endogenous peptides of C1R-A3 cells were first eluted from surface HLA-A3 molecules using a citrate-phosphate buffer and the cells were loaded with the indicated peptides at room temperature for 3 hours. HLA-A3 expression was detected using the GAP-A3 antibody.

[c] Relative binding affinity compared to cells without peptide.

Sequence homology search using the NCBI Blast Database showed that these peptides had high homology to one of the cyclin proteins previously reported to be overexpressed in a variety of epithelial cancers, including breast cancer. To confirm if these peptides were immunostimulatory, we synthesized the peptides and tried to use the original CD8$^+$ T cells

primed with the peptide fractions to see if they responded to the synthetic peptides as well. However, the cells seemed to be on their way to senescence (they have been in culture for 6-7 weeks), because attempts to expand them with either the original tumor or suboptimal anti-CD3 stimulation was unsuccessful. We decided to prime naïve T cells to DCs loaded with the synthetic peptides, and after several rounds of stimulation using autologous macrophages loaded with the synthetic peptides, we tested them using autologous DCs loaded with the peptides in an IFN-γ ELISPOT assay. Our results showed that 2 out of 6 peptides were immunostimulatory (Figure 7). We are now currently in the process of writing a paper covering this aspect of our work. We also plan to sequence the remaining immunostimulatory peptide fractions to find additional breast tumor-specific antigens.

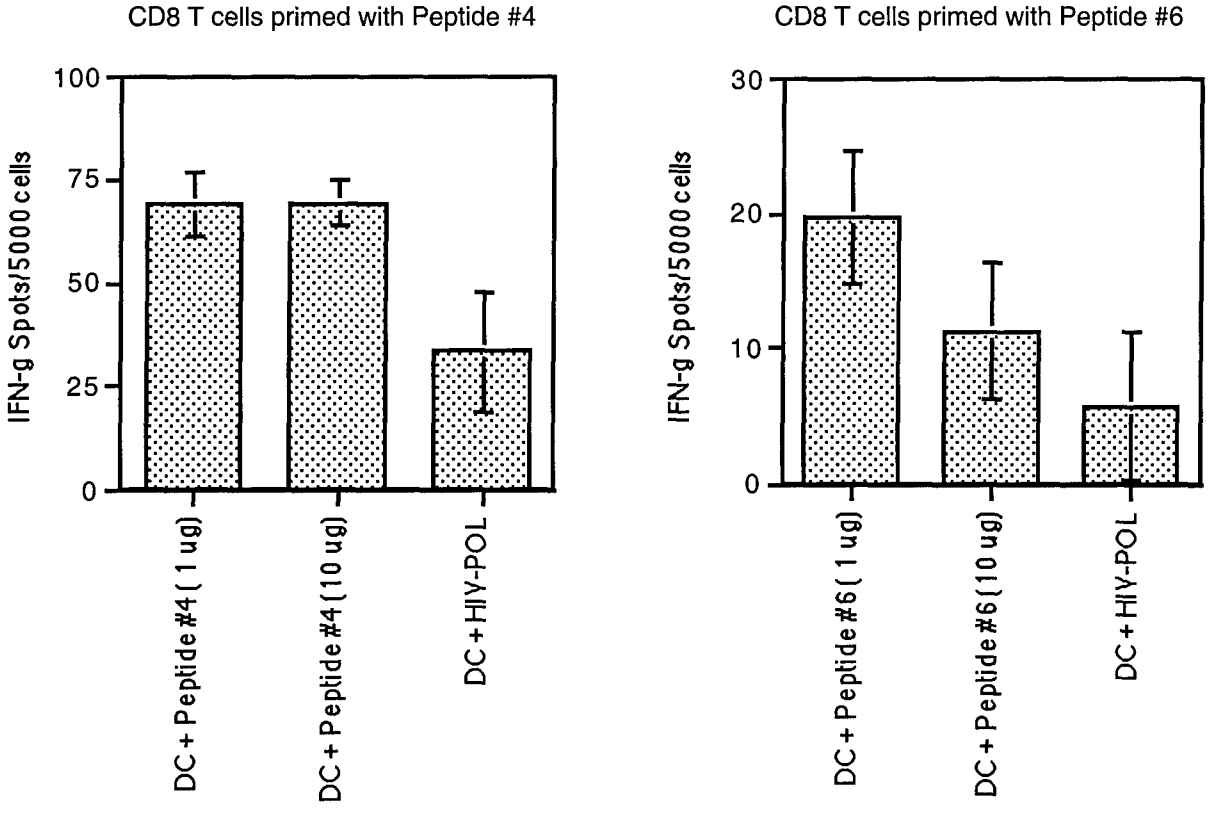

Figure 7. Two peptides identified from our approach contained mmunostimulatory activity. DCs loaded with synthetic peptides were tested against autologous CD8 T cells primed to indicated peptides using an IFN-γ ELISPOT assay.

As mentioned previously (12), we decided last year to focus our attention on the antigen discovery process and place a hold on **Task 3** of this project, which proposes to look at secondary T cell responses from cancer patients to the eluted peptides of the tumor. Recently, however, with the discovery of the tumor peptides described above, we have started to evaluate T cell responses of breast cancer patients to these peptides, thus starting **Task 3** of this project. This will be done using the IFN-γ ELISPOT assay, as well as HLA-A2.1 Class I-tetramers of

these peptides. The results from these ongoing experiments should show conclusively that we have identified a new breast tumor-specific antigen.

Identification of HLA Class II-restricted tumor antigens (Relevant to Technical Objectives 2 &3)

To address **Task 4** of the *Statement of Work*, we generated detergent tumor lysate, which we fractionated by RP-HPLC and loaded onto DCs to prime naïve autologous $CD4^+$ T cells *in vitro*. Details of this work have been described in the DOD '98 Report and will be published in the same manuscript mentioned above. Our results showed that we have been able to obtain the same HPLC profile over different runs (Figure 5B & 5C, ref. 10), allowing us to obtain a constant source of antigen for our studies. We were also able to prime naïve $CD4^+$ T cells to these protein fractions and generate specificity to the tumor, both in apoptotic form (Figure 4) and in tumor lysate form (Figure 5, ref. 11). From this part of the work, we have identified 14 HPLC protein fractions that contain potential breast tumor antigens (Figure 4, ref. 11). We have also subfractionated an immunostimulatory protein fraction from another run, and was able to narrow our search to two predominant proteins of 17 kD and 19 kD (Figure 6, ref. 11). We are currently in the progress of sequencing these two bands, as well as analyzing the remainder 13 positive protein fractions. However, this work has not progressed further due to our exciting results with the HLA Class I-restricted tumor antigens which have become the focus of most of our effort so that we can bring the work to a favorable completion and publication.

KEY RESEARCH ACCOMPLISHMENTS:

- Acid-extraction of peptides bound to tumor HLA Class I molecules
- Reversed-phase HPLC separation of tumor peptides
- Priming of naive $CD8^+$ T cells using autologous dendritic cells loaded with fractionated tumor peptides
- Generation of 12 primed-$CD8^+$ T cell cultures capable of recognizing the original tumor
- Identification of a panel of immunostimulatory peptides by electrospray ionization mass spectrometry analysis
- Confirmation of immunotimulatory capacity in some of the identified peptides
- Reversed-phase HPLC separation of tumor protein extracts
- Priming naive $CD4^+$ T cells using autologous dendritic cells loaded with fractionated tumor proteins
- Generation of 14 primed-$CD4^+$ T cell cultures capable of recognizing the original tumor
- Identification of two immunostimulatory proteins of 17 kD & 19 kD

REPORTABLE OUTCOMES:

Manuscripts:

- Kao, H., Amoscato, A.A., Ciborowski, P., and Finn, O.J. A New Strategy for Tumor Antigen Discovery Based on In Vitro Priming of Naive T Cells with Dendritic Cells. *Clin. Canc. Res.* in press.

Oral Presentations:

- "Priming $CD4^+$ and $CD8^+$ T cells against Tumor Proteins and Peptides using a Dendritic Cell-based Antigen Discovery System"
  Keystone Symposia, Cellular Immunity and Immunotherapy of Cancer, Santa Fe, New Mexico, January 23rd, 2000
- "Identification of MHC Class I-restricted Tumor Peptides Using a Dendritic Cell- Based Antigen Discovery System"
  Pittsburgh Cancer Institute Noon Seminar, January 6th, 2000
- "Priming $CD4^+$ and $CD8^+$ T cells against Epithelial Proteins and Peptides using a Dendritic Cell-based Tumor Antigen Discovery System"
  Fourth Annual University of Pittsburgh Biomedical Student Research Symposium, November 19th, 1999
- "Priming $CD4^+$ and $CD8^+$ T cells against Epithelial Proteins and Peptides using a Dendritic Cell-based Tumor Antigen Discovery System"
  International Conference on Immunology, Shanghai, China, October 26th, 1999
- "Priming $CD4^+$ and $CD8^+$ T cells against Tumor Proteins and Peptides using a Dendritic Cell-based Antigen Discovery System"
  Department of Molecular Genetics & Biochemistry Retreat, September 17th, 1999
- "DC-based Tumor Antigen Discovery System: Work in Progress"
  Pittsburgh Cancer Institute Noon Seminar, September 10th, 1998

Poster Presentations:

- "Priming $CD4^+$ and $CD8^+$ T cells against Epithelial Proteins and Peptides using a Dendritic Cell-based Tumor Antigen Discovery System"
  H. Kao, J. Marto, A. A. Amoscato, P. Ciborowski, J. Shabanowitz, D. Hunt, O. J. Finn
  DOD Era of Hope Meeting, Atlanta, Georgia, June 9-11, 2000
- "Priming $CD4^+$ and $CD8^+$ T cells against Tumor Proteins and Peptides using a Dendritic Cell-based Antigen Discovery System"
  Henry Kao, Andrew A. Amoscato, and Olivera J. Finn
  Keystone Symposia, Cellular Immunity and Immunotherapy of Cancer, Santa Fe, New Mexico, January 21-27th, 2000
- "Priming $CD4^+$ and $CD8^+$ T cells against Epithelial Proteins and Peptides using a Dendritic Cell-based Tumor Antigen Discovery System"
  Henry Kao, Andrew A. Amoscato, and Olivera J. Finn
  Experimental Biology 1999, Washington, D.C. April 17-21, 1999

CONCLUSIONS

For immunotherapies against breast cancer to be sucessful, we must identify breast tumor-specific antigens that could effectively stimulate the immune response of the host. We have devised a novel method for tumor antigen discovery that utilizes the *in vitro* priming of naive T cells with dendritic cells loaded with breast tumor proteins and peptides. This approach has advantages over previous approaches in that it seeks to utilize anti-tumor responses generated from healthy, immunocompetent individuals instead of cancer patients. Furthermore, our approach eliminates the use of tumor cells as stimulators and T cells from cancer patients, since studies have shown that either cell may be inadequate or defective to stimulate an effective

immune response. We believe that by using elements that are "healthy", we can be more effective in identifying new tumor antigens.

For the identification of HLA Class I-restricted tumor antigens, we loaded dendritic cells with peptides eluted from tumor HLA Class I molecules and used it to prime naive $CD8^+$ T cells from a healthy donor. Our results showed that we have been successful in priming T cells to tumor peptides and in generating specificity to the tumor from which the peptides were derived. Mass spectrometry analysis of the immunostimulatory peptides yielded a panel of peptides that could bind HLA Class I molecules, and so far, we have confirmed 2 peptides that bind HLA-A2.1 and contain immunostimulatory capacity. Further work is being done to sequence the remainder 10 peptide fractions to define other new breast tumor-specific antigens.

For the identification of HLA Class II-restricted tumor antigens, we loaded dendritic cells with fractionated tumor lysate and used it to prime naive $CD4^+$ T cells. Our results showed that we have been successful in priming T cells to tumor proteins and generate specificity to the original tumor. Further biochemical analysis of one of the immunostimulatory protein fractions narrowed our search to 2 proteins of 17 & 19 kD. Further work is being done to analyze the remainder 13 protein fractions and define new breast tumor-specific antigens.

In summary, our results show that our approach has been successful in identifying a panel of HLA Class I- and Class II-restricted breast tumor-specific antigens. We are excited at the prospect of this discovery, and currently, we are working to further characterize and define these immunostimulatory tumor peptides and proteins. While the goal of this project was to search for new breast tumor-specific antigens, the universality of our approach allows it to be used to search for new tumor antigens in other types of tumors as well.

REFERENCES

1. Brichard, V., A. Van Pel, T. Wolfel, C. Wolfel, E. De Plaen, B. Lethe, P. Coulie, and T. Boon. 1993. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J Exp Med* 178, no. 2:489.
2. Coulie, P.G., V. Brichard, A. Van Pel, T. Wolfel, J. Schneider, C. Traversari, S. Mattei, E. De Plaen, C. Lurquin, J.P. Szikora, and et al. 1994. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas [see comments]. *J Exp Med* 180, no. 1:35.
3. Hunt, D.F., R.A. Henderson, J. Shabanowitz, K. Sakaguchi, H. Michel, N. Sevilir, A.L. Cox, E. Appella, and V.H. Engelhard. 1992. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry [see comments]. *Science* 255, no. 5049:1261.
4. Hunt, D.F., H. Michel, T.A. Dickinson, J. Shabanowitz, A.L. Cox, K. Sakaguchi, E. Appella, H.M. Grey, and A. Sette. 1992. Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-Ad. *Science* 256, no. 5065:1817.
5. Blake, J., J.V. Johnston, K.E. Hellstrom, H. Marquardt, and L. Chen. 1996. Use of combinatorial peptide libraries to construct functional mimics of tumor epitopes recognized by MHC class I-restricted cytolytic T lymphocytes. *J Exp Med* 184, no. 1:121.

6. Fisk, B., T.L. Blevins, J.T. Wharton, and C.G. Ioannides. 1995. Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. *J Exp Med* 181, no. 6:2109.
7. Tureci, O., U. Sahin, and M. Pfreundschuh. 1997. Serological analysis of human tumor antigens: molecular definition and implications. *Mol Med Today* 3, no. 8:342.
8. Wang, R.F., and S.A. Rosenberg. 1999. Human tumor antigens for cancer vaccine development. *Immunol Rev* 170:85.
9. Finke, J., S. Ferrone, A. Frey, A. Mufson, and A. Ochoa. 1999. Where have all the T cells gone? mechanisms of immune evasion by tumors [In Process Citation]. *Immunol Today* 20, no. 4:158.
10. Kao, H. 1998. Department of Defense Annual Report DAMD17-97-1-7057.
11. Kao, H., A.A. Amoscato, P. Ciborowski, and O.J. Finn. 2000. A New Strategy for Tumor Antigen Discovery Based on In Vitro Priming of Naive T cells with Dendritic Cells. *Clinical Cancer Research*, in press.
12. Kao, H. 1999. Department of Defense Annual Report DAMD17-97-1-7057.

APPENDICES

- Manuscript in press for *Clinical Cancer Research.*

A New Strategy for Tumor Antigen Discovery Based on *In Vitro* Priming of Naïve T Cells With Dendritic Cells [1]

Henry Kao, Andrew A. Amoscato, Pawel Ciborowski, and Olivera J. Finn[2]

Immunology Graduate Program [H.K], Department of Molecular Genetics & Biochemistry [H.K., P.C., O.J.F.], University of Pittsburgh Mass Spectometry Facility, The University of Pittsburgh Center for Biotechnology and Bioengineering [A.A.A.], University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

Key Words: in vitro priming, CD4, CD8

Running Title:

Dendritic Cell-Based Antigen Discovery System

[1] This work is supported by DOD grant DAMD 17-9-1-7057 to H.K. and NIH grant 1PO1CA 73743 to O.J.F.

[2] To whom requests for reprints should be addressed: Olivera J. Finn, University of Pittsburgh School of Medicine, Department of Molecular Genetics & Biochemistry, W1142 Biomedical Science Tower, Terrace & DeSoto Streets, Pittsburgh, PA 15261. Tel: (412) 648-9816, Fax: (412) 383-8859, E-mail: ojfinn@pitt.edu.

ABSTRACT

We describe a method for discovery of new tumor antigens that utilizes dendritic cells as antigen-presenting cells to prime autologous naïve $CD4^+$ and $CD8^+$ T cells from healthy donors against tumor proteins and peptides. For the identification of HLA Class I-restricted tumor antigens, peptides were extracted from tumor HLA Class I molecules, fractionated by RP-HPLC, and loaded onto *in vitro* generated DCs to prime naïve $CD8^+$ T cells. Our results show that we were able to prime naïve $CD8^+$ T cells *in vitro* to several peptide fractions and generate specificity for the tumor. Electrospray ionization mass spectrometry was used to confirm that these fractions contained peptides derived from MHC-Class I molecules and the primed $CD8^+$ T cells were used to further analyze the immunostimulatory peptide fractions. For the identification of HLA Class II-restricted tumor antigens, we fractionated tumor protein extracts using RP-HPLC, and loaded individual fractions onto DCs to prime naïve $CD4^+$ T cells. Our results show that we were also able to prime naïve $CD4^+$ T cells to several protein fractions and generate specificity for the tumor. These results illustrate the potential of this method to identify new immunostimulatory MHC Class I- and Class II-restricted tumor antigens.

INTRODUCTION

The prospect of successful immunotherapy against tumors relies in part on the discovery of tumor-specific antigens that are able to stimulate effective immune responses in the host. Several approaches have been developed over the years for the identification of tumor antigens. The "genetic approach" utilizes tumor cDNA libraries transfected into target cells expressing appropriate HLA molecules (1, 2). The "peptide elution approach" utilizes peptides eluted from tumor HLA and loaded on target cells bearing the same HLA molecules (3, 4). The "reverse immunology approach", utilizes peptide sequences derived from already known oncogenes or other putative tumor associated genes that contain desired HLA anchor motifs (5, 6). All these approaches depend on the availability of tumor-specific T cell lines or clones derived from cancer patients and used to recognize the new targets. Most recently, the SEREX approach has been used where tumor cDNA expression libraries are screened with sera from cancer patients (7). These approaches have led to the identification of a panel of tumor antigens, primarily in melanomas (8-15). Very few tumor-specific antigens have been described in epithelial tumors, the best known being the HER-2/neu derived peptides (16), and the core peptides of the MUC-1 tandem repeat (17). In addition to the overall number of tumor antigens being small, there is a concern that they were all identified using an immune response from individuals with cancer that has clearly not been effective as a tumor rejection response. Therefore, we devised and tested a new antigen discovery system that seeks to determine not what a cancer patient recognizes on his/her tumor, but rather, what a healthy immune system recognizes as foreign among numerous peptides and proteins derived from tumor cells. Our approach is based on *in vitro* cultured dendritic cells that are used to prime autologous healthy naïve T cells.

Dendritic cells have been shown to be the most potent APCs in the immune system, expressing high levels of MHC molecules, costimulatory molecules and adhesion molecules essential for T cell activation (18). Furthermore, dendritic cells are capable of inducing primary T cell responses *in vitro* to both viruses and synthetic peptides (19-22), whereas other APCs[3] can only stimulate previously sensitized T cells (23). DCs have been sucessfully used to prime naïve T cells *in vitro* against several known tumor antigens (24-30).

Studies have shown marked alterations in the signal-transduction molecules in the T cells of human cancer patients, such as alterations in expression of $p56^{lck}$ tyrosine kinase, TCR-ζ chain (31), and NF-κB p65 transcription factors (32). Therefore, in our approach we have used T cells from healthy donors. The results here illustrate the utility of this new approach for priming naïve $CD4^+$ and $CD8^+$ T cells against proteins and peptides isolated from a breast epithelial tumor cell line and presented by DCs grown *in vitro*, and generation of specificity for the tumor. This has lead to the isolation of candidate HLA Class I- and HLA Class II-restricted epithelial tumor antigens.

[3] The abbreviations used are: APC, antigen presenting cell; DC, dendritic cell; RP-HPLC, reverse-phase high performance liquid chromatography; TFA, trifluoroacetic acid; PBMC, peripheral blood mononuclear cells; CTL, cytotoxic T lymphocytes

MATERIALS AND METHODS

Cell lines. MS (A3, B7, B7, C7, C7; DR15, DQ6 homozygous) is a breast epithelial adenocarcinoma cell line derived from the metastasis of a breast cancer patient. This cell line does not express either MUC-1 or HER-2/neu (data not shown), the two major epithelial tumor antigens. MS-A2 is the same cell line that we stably transfected with the HLA-A2.1 plasmid (33) using the calcium phosphate precipitation method (Stratagene, La Jolla, CA). The B lymphoma cell line Raji (A3, B15, C7 homozygous; DR3, DR10, DQ1, DQ2) was purchased from the American Type Culture Collection (Manassas, VA). The chronic myelogenous leukemia cell line K562 was also purchased from ATCC. The melanoma cell line Mel 624 (A2, A3, B7 homozygous) was a kind gift from Dr. Walter Storkus, University of Pittsburgh. The lung tumor cell line, 201T (A10, A29, B15, B44), which we also transfected with the HLA-A2.1 plasmid (designated 201T-A2), was a kind gift from Dr. Jill Siegfried, University of Pittsburgh. Naïve $CD4^+$, $CD8^+$ T cells, dendritic cells, and macrophages were derived from a leukophoresis product of a healthy platelet donor (A2, A29, B7, B44, C7, C7; DR15, DR7, DQ6, DQ2) obtained through the Central Blood Bank of Pittsburgh (Pittsburgh, PA).

Antibodies. Mouse anti-human HLA-DR (L243), CD3 (Leu-4), CD4 (Leu-3a), CD8 (Leu-2a), and CD56 (Leu-19) were purchased from Becton Dickinson (San Jose, CA). Mouse anti-human CD45RO (UCHL1) and CD20 were purchased from DAKO (Carpinteria, CA). Goat anti-mouse IgG antibodies were obtained from Zymed Laboratories, Inc. (South San Francisco, CA). W6/32, a mouse anti-human MHC Class I antibody, was produced by the W/632 hybridoma obtained from ATCC (Manassas, VA) and purified via a Protein A-Sepharose column (Sigma, St. Louis, MO) in the laboratory.

Isolation of tumor HLA Class I-bound peptides. Isolation of HLA Class I-associated peptides was similar to previously described methods (3, 34). MS-A2 tumor cells were grown in 10-chamber cell factories (Nalge Nunc, Naperville, IL), and expanded weekly until >1.5 x 10^{10} cells were obtained. The cells were washed three times in ice-cold PBS, pelleted and stored at -80^0 C for later use. Detergent lysis buffer (1% CHAPS) and a cocktail of protease inhibitors (2 mM PMSF, 100 μM Iodoacetamide, 5 μg/ml Aprotinin, 10 μg/ml Leupeptin, 10 μg/ml Pepstatin A, 3 ng/ml EDTA, and 0.04% sodium azide) (Sigma, St. Louis, MO) were used to solubilize the cells at 4°C for 1 hr. The cell lysate was spun at 100,000 x g for 1 hour to remove insoluble proteins, and the supernatant was filtered through a 0.22 μm filter (Millipore, Bedford, MA) to further remove debris from the suspension. The supernatant was then passed through a Protein A-Sepharose anti-class I (W6/32) column (BioRad, Hercules, CA) overnight. The column was then washed 30 times sequentially with low salt (150 mM NaCl, 20 mM Tris pH 8.0) buffer, high salt (1 M NaCl, 20 mM Tris pH 8.0) buffer, and Tris buffer (20 mM Tris pH 8.0). Class I molecules were then eluted from the column using 0.2N acetic acid, and peptides were extracted from the Class I molecules by boiling in 10% acetic acid for 5 minutes. The released peptides were further purified using 5 kD cut-off microconcentrators (Amicon, Bedford, MA), vacuum centrifuged to reduce the volume, and frozen at -80^0C.

High Performance Liquid Chromatography. 1) Fractionation of peptide extracts. The peptide extracts were fractionated by reverse phase HPLC on a Rainin HPLC separation system (Varian, Woburn, MA). The peptide extracts were concentrated to 150 μl via vacuum centrifugation, and injected into a Brownlee Aquapore (Applied Systems Inc., San Jose, CA) C18 column (column dimensions: 2.1mm x 3 cm, pore size: 300 Å, particle size: 7 μm) on the HPLC. The peptides

were eluted with a 65 minute trifluoracetic acid/acetonitrile gradient [v/v 0-15% for 5 minutes, 15-60% for 50 minutes, and 60-100% for 10 minutes solvent B (60% acetonitrile in 0.085% TFA) in solvent A (De-ionized water in 0.1% TFA)] with a flow rate of 200 µl/min. Two hundred microliter fractions were collected at one minute intervals, concentrated via vacuum centrifugation to 40 µl, and divided into 4 aliquots, 3 for the use in T cell stimulation and 1 for mass spectrometry analysis.

2) Fractionation of protein extracts. >1 x 10^9 MS tumor cells were lysed in detergent buffer, spun at 100,000 x g, and then filtered using a 0.22 µm filter as above. The supernatant was dialyzed overnight in Tris Buffered Saline pH 7.2 (TBS) (Sigma) to remove detergent. The protein extract was concentrated by vacuum centrifugation, and one-tenth of the extract (~1 x 10^8 cell equivalents) was fractionated by reverse-phase HPLC using a Phenomenex Jupiter C4 column (column dimensions: 4.6 mm x 150 mm, pore size: 300 Å, particle size: 7 µm) (Torrence, CA). The proteins were eluted with a 60 minute TFA/acetonitrile gradient [v/v 10-80% solvent B (100% acetonitrile in 0.1 TFA) in solvent A (De-ionized water in 0.1% TFA)] at a flow rate of 500 µl/min. Five hundred microliter fractions were collected at one minute intervals, concentrated by vacuum centrifugation to 100 µl, and divided into 4 aliquots, 3 for the use in T cell stimulation, and 1 for protein content analysis.

3. Sub-Fractionation of protein fractions. 25% of a specific protein fraction obtained from the primary fractionation was further sub-fractionated by reverse-phase HPLC using a Phenomenex Jupiter C4 column (column dimensions: 4.6 mm x 150 mm, pore size: 300 Å, particle size: 7 µm) (Torrence, CA). The proteins were eluted with a shallow 10 minute TFA/acetonitrile gradient [v/v 55-62% solvent B (100% acetonitrile in 0.1 TFA) in solvent A (De-ionized water in 0.1% TFA)] at a flow rate of 500 µl/min, and fractions were collected at one minute intervals.

The sub-fractions were then further concentrated by vacuum centrifugation, with 33% of the material loaded onto a 15% SDS-PAGE gel and visualized using a silver stain analysis kit (BioRad), and 33% was loaded onto macrophages and used in a proliferation assay (see below). All solvents were HPLC grade and were obtained from VWR Scientific Products (West Chester, PA).

Generation of DCs *in vitro*. DCs were cultured *in vitro* for 7 days as described previously (28). PBMCs from a healthy donor were isolated after centrifugation over Lymphocyte Separation Medium™ (Organon Teknika, Durham, NC) and washed extensively with PBS to eliminate residual platelets. The cells were plated in a T-75 flask for two hours in serum-free AIM-V (Life Technologies, Grand Island, NY) medium, after which the non-adherent cells were removed and used as the source of naïve T cells (see below). The adherent cells were treated with 1000 U/ml GM-CSF and 26 ng/ml IL-4 (Schering Plough, Kenilworth, NJ) for 7 days in serum-free AIM-V (Life Technologies) medium supplemented with 2 mM L-glutamine and penicillin/streptomycin. DCs were fed with additional media and cytokines on Day 4 of culture, and purified on Day 7 by negative selection of contaminating T, B, and NK cells. The cells were stained with anti-CD3, -CD19, and -CD56 antibodies for 45 minutes in cold PBS and washed in PBS supplemented with 5% human AB serum (Gemini Products, Calabasas, CA). Magnetic DYNAL™ beads (Lake Success, NY) coated with goat anti-mouse IgG were then added to the cells for 45 minutes, and the contaminating cells were removed by magnetic separation. Flow cytometry analysis of the remaining cells showed they were high HLA-DR$^+$ and B7-2$^+$.

Purification of naïve $CD8^+$ and $CD4^+$ T cells. Non-adherent cells obtained after plastic adherence for DC isolation was used as the source of naïve $CD8^+$ or naïve $CD4^+$ T cells. To purify naïve $CD8^+$ T cells, the cells were stained with anti-CD4, -CD20, CD56, and CD45RO antibodies for 45 minutes in cold PBS and washed in PBS with 5% HAB serum. Four 100-mm petri dishes (Nunc LabTek, Naperville, IL) were precoated with 10 μg/ml goat anti-mouse IgG antibodies (Zymed) in 0.05 M Tris, pH 9.5 at room temperature for 1 hr and washed with PBS. Cells were added to each plate and incubated at 4^0C for 1 hr. The non-adherent cells collected were the $CD45RA^+CD8^+$ T cells. The same procedure was used for purifying naïve $CD4^+$ T cells, except that anti-CD8 antibodies are used instead. All T cell cultures were grown in RPMI medium (ICN, Costa Mesa, CA) supplemented with 10% human AB sera (Gemini Products), L-glutamine, and penicillin/streptomycin (Life Technologies).

Priming naïve $CD8^+$ T cells to tumor peptides. To prime naïve $CD8^+$ T cells, 2×10^4 dendritic cells were incubated for 2-4 hours first with 25% of each peptide containing RP-HPLC fraction (10 μl), and then overnight in the presence of 1000 U/ml TNF-α (Genzyme, Cambridge, MA) in 96-well U-bottom plates (Falcon, Franklin lakes, NJ). 2×10^5 autologous naïve $CD8^+$ T cells were added the next day to the DCs in the presence of 2 ng/ml IL-1β (R & D Systems, Minneapolis, MN), 20 U/ml IL-2 (DuPont, Wilmington, DE), and 26 ng/ml IL-4 (Schering Plough). The $CD8^+$ T cell cultures were fed every 3-4 days with 10 U/ml IL-2 and 13 ng/ml IL-4, depending on growth kinetics. In addition, 10 ng/ml IL-7 (Pharmingen, San Diego, CA) was included in the cytokine mixture after the 2^{nd} restimulation. The $CD8^+$ T cell cultures were restimulated every 7-10 days using autologous macrophages (obtained by plastic adherence) loaded with the individual peptide fractions, until the 3^{rd} restimulation, where autologous

macrophages loaded with irradiated (12, 000 Rads) MS-A2 tumors (macrophages : tumor = 1 : 5) were used as stimulators (T cells : loaded macrophages = 10 : 1).

Priming naïve $CD4^+$ T cells to tumor proteins. To prime naïve $CD4^+$ T cells, dendritic cells were loaded with 25% of each protein fraction (~ 25 μl), and treated the same as described for $CD8^+$ T cell priming above. The $CD4^+$ T cell cultures were restimulated every 10-14 days using autologous macrophages loaded with the individual protein fractions (T cells: macrophages = 10:1), and fed every 4-5 days with 10 U/ml IL-2 and 13 ng/ml IL-4, depending on growth kinetics. 10 ng/ml IL-7 (Pharmingen) was added to the $CD4^+$ T cell cultures after the 2^{nd} restimulation. For the 3^{rd} restimulation, MS tumor was irradiated for 7 minutes (2.18 J/cm^2) using a Spectra Mini II UV-B irradiator (Daavlin, Bryan, Ohio) and loaded onto macrophages overnight (5:1 = apoptotic tumor : macrophages) that were used as stimulators (T : loaded macrophages = 10:1) the next day.

Cytotoxicity assays. 1-2 x 10^6 target cells were labeled with 50 μCi of $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, IL) for 90 minutes at 37^0C. The labeled cells were then washed three times and plated at 1 x 10^3 cells/well in a 96-well V-bottom plate (Costar, Cambridge, MA) with various numbers of effector T cells. In addition, a 50-fold excess of unlabeled K562 (5 x 10^4) was added to the wells for 15 minutes prior to the addition of T cells to prevent the detection of lymphokine-activated killer (LAK) activity in the assay. The plates were centrifuged and incubated for 4 hours at 37°C. All determinations were done in triplicate. Supernatants were harvested using a Skatron harvesting press (Skatron Instruments, Sterling, VA) and counted on a Cobra II series auto gamma counting system (Packard, Meriden, CT). Maximum release was

obtained by adding 50 µl of 1% Triton X-100 to the labeled target cells. Spontaneous release was obtained by incubating the labeled cells in the absence of T cells. Percent specific lysis was calculated from the following formula: % specific lysis = 100 x (experimental release - spontaneous release)/(maximum release - spontaneous release). In blocking experiments, anti-MHC Class I Ab (W6/32) was added to the labeled target cells for 30 minutes prior to the addition of the effector T cells.

Proliferation Assays. Autologous macrophages loaded with UV-induced apoptotic MS tumor cells (5:1 = apoptotic tumor : macrophages) were seeded in round-bottom 96-well microplates (Costar, Cambridge, MA) with primed T cell cultures at a T cell : stimulator ratio of 20 : 1. For proliferation assays using tumor lysate, MS tumor cell lysate was generated as described previously, and 1.75×10^8 cell equivalents were loaded onto 2×10^6 autologous macrophages for 2 hours. T cells were added at a T cell : stimulator ratio of 10 : 1. For the proliferation assay using the sub-fractions of #44, 33% of the sub-fraction was loaded onto 5×10^4 macrophages overnight and added to T cells with a T : stimulator ratio of 1:1 the next day. The wells were pulsed with [^{3}H]TdR (Amersham, Life Science) for the last 18 hours of the 5-day period, harvested by a Skatron semiautomatic cell harvester (Skatron Instruments), and counted on a Wallac 1205 beta plate scintillation counter (Gaithersburg, MD). The results are expressed as mean values of triplicate determinations.

Mass spectrometry analysis. 25% of the RP-HPLC peptide fraction was concentrated by vacuum centrifugation to near dryness and resuspended in 5 µl of 0.1M acetic acid. One microliter of this material was loaded onto a microcapillary C18 column (150 mm x 75 µm i.d.),

and eluted with a 20 minute linear gradient [v/v 0-80% solvent B (0.1 M acetic acid in 100% acetonitrile) in solvent A (De-ionized water in 0.1 M acetic acid)]. Flow rates for the nanospray probe (186 nl/min) was achieved by coupling the Rainin HPLC system with an Accurate microflow processor (LC Packings, San Francisco, CA) for flow splitting. The nanospray probe was operated at a voltage differential of +3.2 keV. The source temperature was maintained at 30^0C. Mass spectra were obtained by scanning from 300-1500 every 3 seconds and summing individual spectra on a Fisons Quattro II triple quadrupole mass spectrometer (Micromass Inc., Loughborough, U.K.).

RESULTS

Identification of HPLC fractions containing immunogenic tumor peptides. $CD8^+$ T cell cultures were primed and restimulated with HPLC fractions as described in the *Materials & Methods*. Due to the low amount of peptide, later restimulations were done using macrophages loaded with irradiated MS-A2 tumor cells. Monitoring the $CD8^+$ T cell cultures with an inverted microscope over four restimulations clearly showed that while there was T cell proliferation in all wells, several of the $CD8^+$ T cell cultures were expanding at a much higher rate, suggesting the presence of immunostimulatory peptides in the fractions used for priming in these wells. Most of the unstimulated $CD8^+$ T cell cultures reached senescence after 8 weeks in culture.

Figure 1 shows the result of one priming experiment in which after the 4^{th} restimulation, we were able to test all the T cell cultures for their ability to recognize the original tumor, MS-A2, from which the peptides were derived. Out of the 65 $CD8^+$ T cell cultures primed on the individual peptide fractions, 12 (Fractions 15, 22, 30, 32, 37, 38, 43, 44, 50, 51, 52, 63) exhibited strong cytotoxicity against the tumor. Since the PBL donor and the tumor were mismatched at the HLA-A3 allele, we also used the Raji tumor cell line, which was matched only at the HLA-A3 allele with the PBL donor, to ensure that the cytotoxic $CD8^+$ T cell cultures were not alloreactive. None of the cultures that killed MS-A2 tumor cells recognized the Raji targets.

The 12 cytotoxic $CD8^+$ T cell cultures were also tested for their ability to recognize another epithelial adenocarcinoma, a lung tumor, 201T-A2, to look for shared tumor antigens. As shown in Figure 2, a $CD8^+$ T cell culture, primed with peptide fraction #32, recognized again the original tumor, and also the lung tumor. Since the lung tumor and the breast tumor shared

only the HLA-A2.1 allele, this suggested that the peptide being recognized was a shared antigen restricted by HLA-A2.1.

To determine the extent of reproducibility of this approach, we repeated the acid extraction, peptide fractionation, and priming procedure. We decided to pool several consecutive HPLC fractions for two reasons: 1) to compensate for small shifts in fraction number between HPLC runs, and 2) to reduce the total number of T cell cultures *in vitro,* making the approach less labor intensive. Naive $CD8^+$ T cells were primed on pooled fractions that were composed of the immunostimulatory fractions identified earlier as well as flanking fractions. The patterns of T cell stimulation and expansion induced by the pooled fractions were consistent with the patterns induced by the immunostimulatory fractions observed in the previous priming. An example is shown in Figure 3, in which we primed $CD8^+$ T cell cultures to pooled fractions 41-46 (Figure 3A) and 61-65 (Figure 3B), and generated specificity to the original tumor, MS-A2. This corresponded to immunostimulatory fraction # 44 and fraction # 63 from the previous run, respectively (Figure 1). We also showed that $CD8^+$ T cells primed on pooled fractions 41-46 were blocked by the anti-MHC Class I antibody, W6/32 (Figure 3A). Furthermore, we showed that the specific antigen was present in the epithelial tumor, and not in the perfectly HLA-matched melanoma, Mel 624 (Figure 3B).

To evaluate the content of these 12 immunostimulatory fractions, we analyzed them by electrospray ionization nanospray mass spectrometry. We obtained a panel of peptide species (Table 1) conforming to mass-to-charge ratios of 700-1300 Daltons, indicative of HLA Class I-binding peptides. These results showed that we were successful in extracting peptides from HLA Class I molecules, and that there were immunostimulatory peptides in the HPLC fractions that were capable of stimulating naïve $CD8^+$ T cells to proliferate and expand *in vitro*. We are

in the process of sequencing these peptides, and so far, we have identified a series of peptides that belong to a new tumor antigen (Kao et al., manuscript in preparation).

Identification of HPLC fractions containing immunogenic tumor proteins. We have devised and tested a similar strategy to identify HLA Class II-restricted tumor antigens by analyzing immunostimulatory properties of fractionated tumor proteins. $CD4^+$ T cell cultures were primed and restimulated as described in the *Materials & Methods*. By the 3^{rd} restimulation, macrophages loaded with apoptotic MS tumor cells were used to stimulate the $CD4^+$ T cell cultures. Similar to the $CD8^+$ T cell cultures, observation of the $CD4^+$ T cell cultures with an inverted microscope over five restimulations showed that not all the $CD4^+$ T cell cultures were growing equally well, suggesting that the $CD4^+$ T cells were responding to immunostimulatory proteins present in some of these fractions, and not in others. Most of the unstimulated $CD4^+$ T cell cultures reached senescence after 10 weeks in culture.

Figure 4 shows the results of one priming experiment in which after the 2^{nd} restimulation, we tested all the T cell cultures for their ability to recognize the original tumor, MS, from which the proteins were obtained. Autologous macrophages were loaded with apoptotic tumor and used in a 5-day proliferation assay as stimulators of the primed $CD4^+$ T cell cultures. Out of the 52 $CD4^+$ T cell cultures primed on the individual protein fractions, 14 (Fractions 5, 10, 11, 12, 13, 22, 28, 35, 37, 38, 39, 40, 46, 51) proliferated in response to macrophages loaded with apoptotic tumor. We also tested the $CD4^+$ T cell cultures for cytotoxicity against the original tumor via a CTL assay. None of the T cells tested killed the original tumor (data not shown). Some of the positive $CD4^+$ T cell cultures were also tested for their ability to recognize autologous macrophages loaded with tumor lysate. As shown in

Figure 5, $CD4^+$ T cell cultures primed with protein fractions #5-6 and #15 were able to proliferate to macrophages loaded with tumor lysate, consistent with results shown in Figure 4.

To determine the content of the immunostimulatory protein fractions, we evaluated the protein fractions using SDS-PAGE and silver stain analysis. Because of a large number of proteins in each fraction, the fractions of interest were further subfractionated by RP-HPLC. An example is shown in Figure 6 where we analyzed the immunostimulatory protein fraction # 44 from the second HPLC run that corresponded to fraction #46 from the first run (Figure 4), due to slight variations in HPLC fraction number between runs. From fraction #44 we derived 10 sub-fractions, and analyzed them for protein content (Figure 6A) and immunostimulatory capacity (Figure 6B). We detected immunostimulatory capacity in 4 of the 10 subfractions (#44.1, #44.2, #44.4, and #44.7). Silver stain analysis detected two prominent bands, one at 17 kD, and the other at 19 kD in fraction 44.4 (Figure 6A). We are in the process of sequencing these bands, as well as determining the immunostimulatory proteins in the other HPLC fractions.

DISCUSSION

We have devised and tested a new approach to tumor antigen discovery that utilizes dendritic cells as antigen-presenting cells to prime naive $CD4^+$ and $CD8^+$ T cells against tumor

proteins and peptides. This approach has advantages over previous approaches in that it seeks to explore anti-tumor responses that can be generated in healthy, immunocompetent individuals, to tumor antigens presented by professional APCs. This new approach eliminates the use of T cells from cancer patients and the use of tumor cells as APCs. Studies have shown that T cells in cancer patients may be defective, and tumor cells are poor APCs (35, 36). In addition, by using T cells from healthy donors, we are relying on a T cell repertoire that has not been affected by the presence of the tumor.

We have used this approach in search of new epithelial tumor antigens which have been limiting to date mainly due to the difficulty in generating tumor cell lines and consequently tumor-reactive T cell lines (8). For the identification of HLA-Class I-restricted tumor antigens, we showed that we were able to prime naive $CD8^+$ T cells to tumor peptides and generate specificity to the original tumor from which the peptides were derived. In some cases, the primed $CD8^+$ T cells also recognized another tumor of the same tissue type, suggesting the existence of shared tumor antigens. We also showed that we could reproduce the whole procedure, demonstrating that there were indeed tumor peptides present in similar fractions that conferred immunostimulatory capacity.

To determine the composition of immunostimulatory peptide fractions, we used electrospray ionization mass spectrometry to look for masses that would conform to what is expected of HLA Class I-associated peptides which have mass-to-charge ratios between 700-1300 Daltons. The advantage of using the mass spectrometer to identify HLA Class I-associated peptides have been extensively documented (37). It offers high sensitivity for detection of small amounts of peptides. We were able to identify a panel of peptides that fit the criteria of HLA Class I-binding peptides, suggesting that these immunostimulatory fractions contained peptides

that could be MHC Class I-restricted candidate tumor antigens. The identification and sequencing of these naturally processed peptides is currently ongoing. Fraction #50 (Figure 1) has already yielded a new tumor antigen (Kao et al., manuscript in preparation) not found by other approaches to date.

For the identification of HLA Class II-restricted tumor antigens, we demonstrated that we were able to prime naive $CD4^+$ T cells to fractionated tumor proteins and generate specificity against macrophages fed the original tumor, both in the apoptotic form and the tumor lysate form. Not surprisingly, SDS-PAGE and silver stain analysis of each of the positive protein fractions yielded an array of protein bands (data not shown), requiring further sub-fractionation and confirmation of immunostimulatory capacity. Sub-fractionation of one of these protein fractions yielded two predominant proteins of 17 kD and 19 kD, narrowing our search to two specific tumor antigen candidates. Investigations are currently underway in our lab to further sub-fractionate and sequence other positive protein fractions identified by *in vitro* priming of $CD4^+$ T cells.

Although we expect that this new approach will be capable of identifying numerous new tumor antigens, it is not without its disadvantages. As all the other previously used methods, it is very labor intensive. Furthermore, it is very dependent on the ability to grow and expand tumor peptide or tumor protein-specific T cells. In our experience, we have been able to generate only 3-5 $x10^6$ $CD8^+$ and $CD4^+$ T cells per HPLC fraction, which severely limited the number of cytotoxicity and proliferation assays that could be done to evaluate their specificity. This problem is becoming less critical, however, with the advent of new functional assays that use very few T cells, such as ELISPOT (38). The technical complexities of using the mass spectrometer to detect and sequence peptides from these fractions could also be a daunting task,

although we were moderately successful in identifying a panel of peptides at the 100 femtomole level.

In summary, by devising a system that relies on healthy donors, we have utilized the full immune potential of the donor's T cells and DCs as reagents to screen for new tumor antigens, and we have detected a number of candidate tumor antigens that are being further characterized. While we focused our search on epithelial tumor antigens, the strength of our approach is its universal applicability to all tumors, including those that do not readily grow *in vitro*. While we have utilized a tumor cell line as a source of peptides and proteins, they can just as easily be derived from pieces of tumor removed at the time of surgery. And lastly, in this approach the peptides and proteins that are found to be immunostimulatory are already known to be naturally processed and presented, an important factor in the design of cancer vaccines.

ACKNOWLEDGMENTS

We would like to thank Robert A. Henderson and Elizabeth M. Hiltbold for their help at the beginning of this project. We would also like to thank Joseph Ahearn for sharing his flow cytometer and UV-irradiator.

BLANK

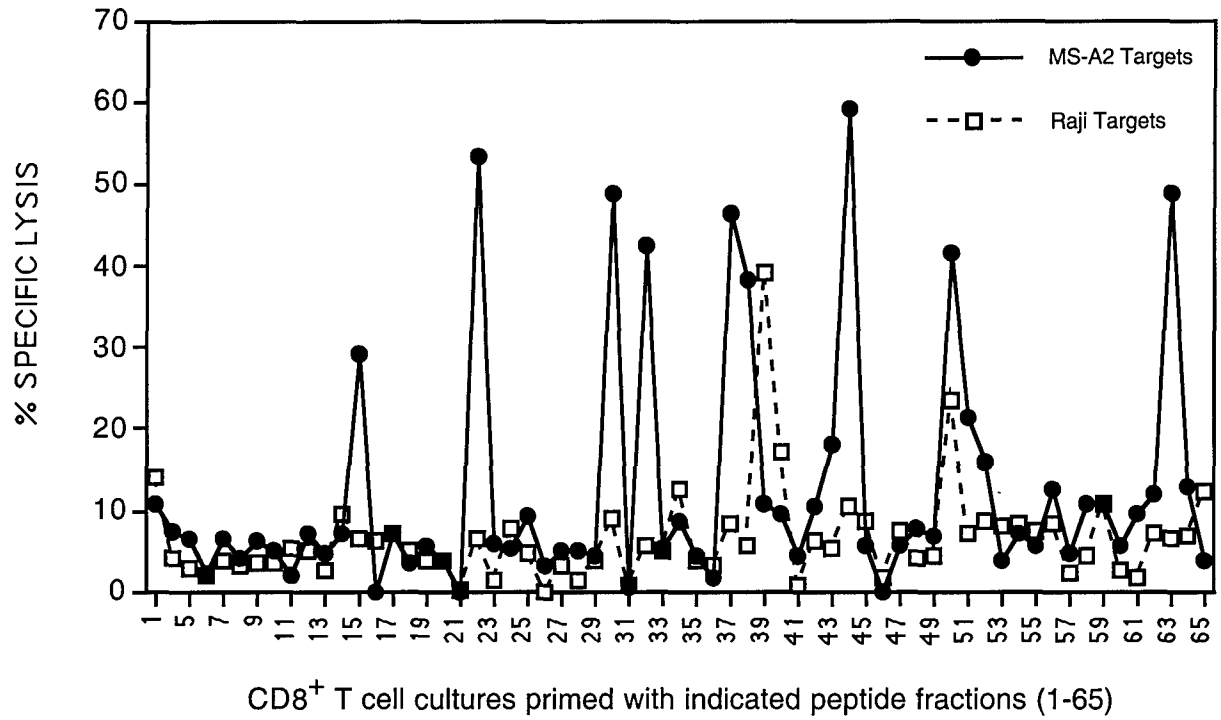
70
60
50
40
30
20
10
0
% SPECIFIC LYSIS
MS-A2 Targets
Raji Targets
1
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
37
39
41
43
45
47
49
51
53
55
57
59
61
63
65
CD8+ T cell cultures primed with indicated peptide fractions (1-65)

Figure 1

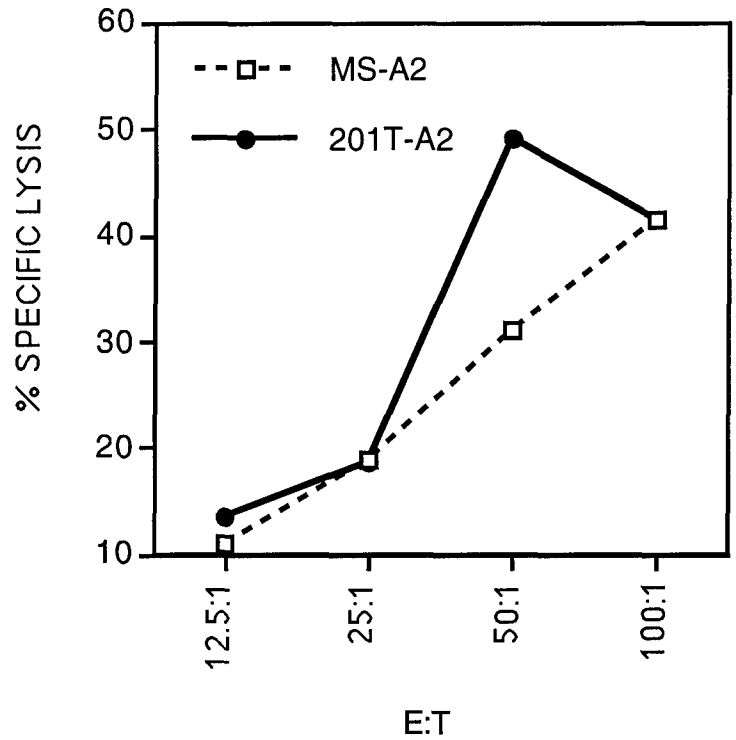
60
50
40
30
20
10
% SPECIFIC LYSIS
MS-A2
201T-A2
12.5:1
25:1
50:1
100:1
E:T

Figure 2

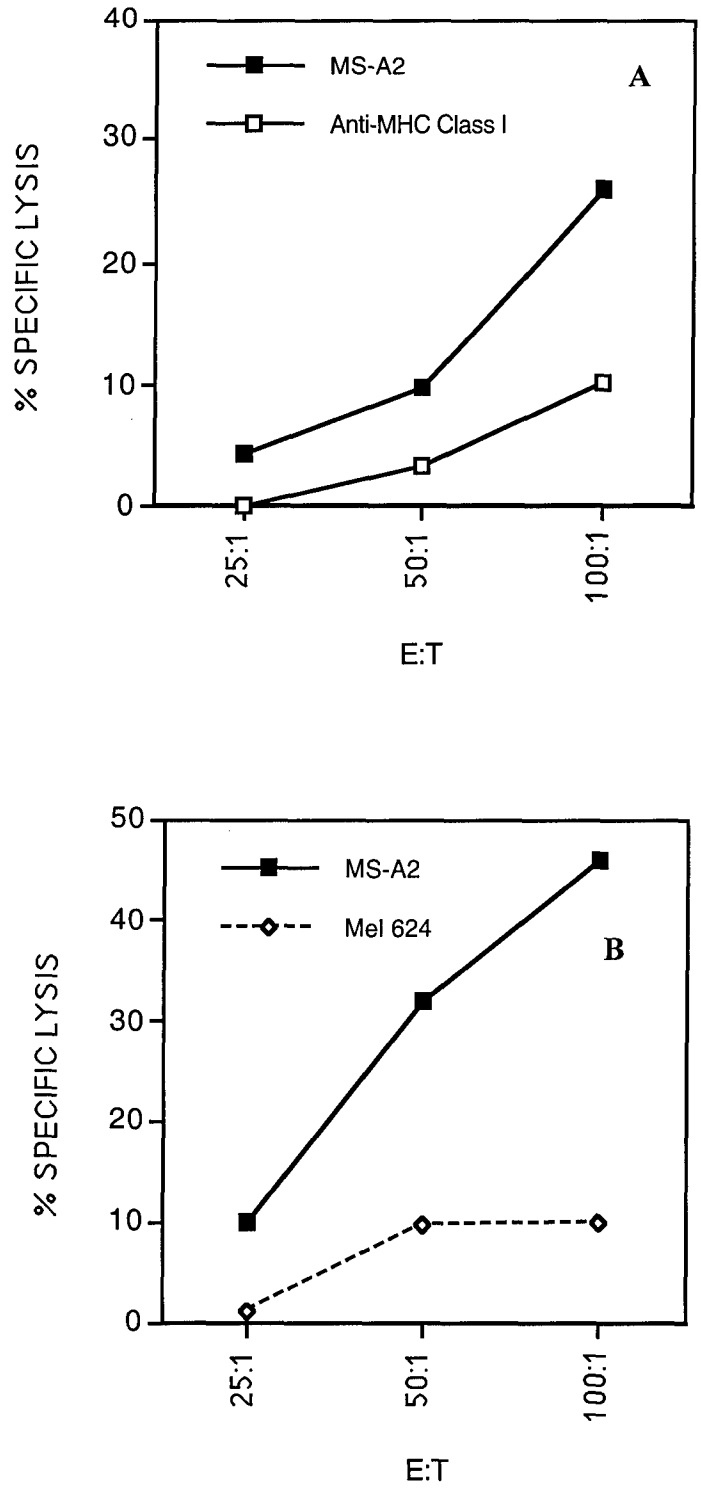
40
30
20
10
0
% SPECIFIC LYSIS
MS-A2
Anti-MHC Class I
A
25:1
50:1
100:1
E:T
50
40
30
20
10
0
% SPECIFIC LYSIS
MS-A2
Mel 624
B
25:1
50:1
100:1
E:T

Figure 3

Table 1: Mass spectrometry analyses of immunostimulatory peptide fractions[a].

Fraction #	m/z[b]	Fraction #	m/z
15	851.7, 879.8 [c]	44	615.2, 1229.5, 942.3, 921.4, 1061.5
22	921.3, 1061.4	50	728.1, 949.2, 1256.5
30	717	51	949
32	717	52	949, 885.9
37	None	63	805.6
38	921.3		
43	949.2, 816		

[a] HPLC peptide fractions that tested positive in the CTL assay were analyzed by nanospray microcapillary HPLC mass spectrometry.

[b] Mass-to-charge ratio.

[c] Peptide mass to charge ratios (m/z) conforming to peptides that bind HLA Class I molecules (700-1300 Daltons) were considered candidates for tumor antigens.

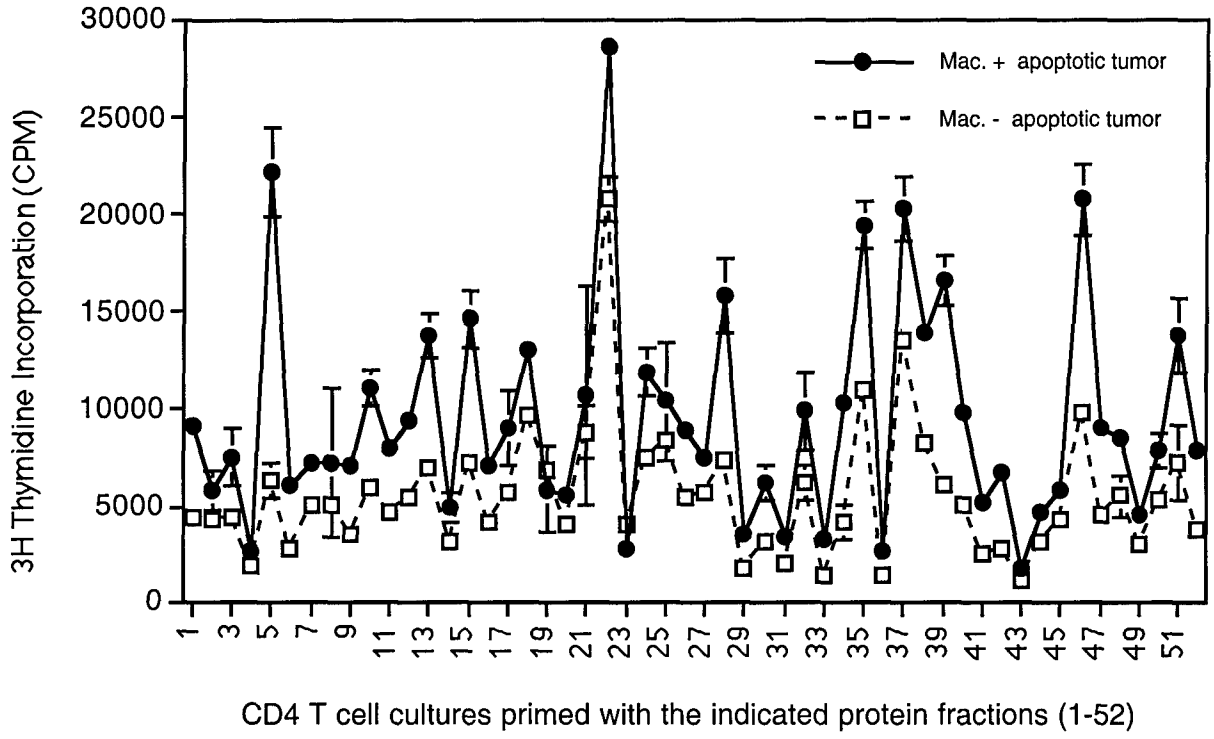
30000
25000
20000
15000
10000
5000
0
3H Thymidine Incorporation (CPM)
Mac. + apoptotic tumor
Mac. - apoptotic tumor
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
37
39
41
43
45
47
49
51
CD4 T cell cultures primed with the indicated protein fractions (1-52)

Figure 4

Figure 5

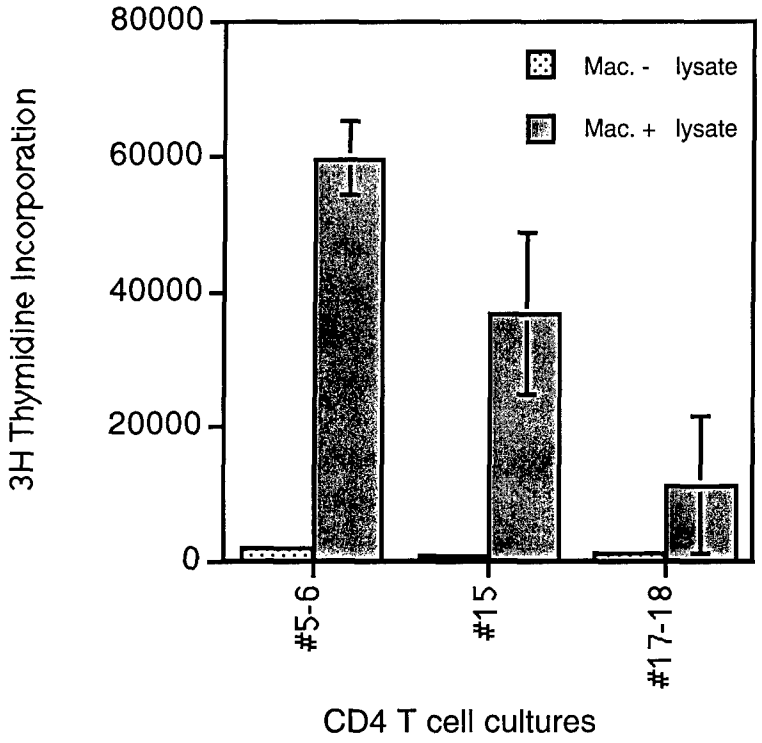
80000
60000
40000
20000
0
3H Thymidine Incorporation
Mac. - lysate
Mac. + lysate
#5-6
#15
#17-18
CD4 T cell cultures

Figure 6

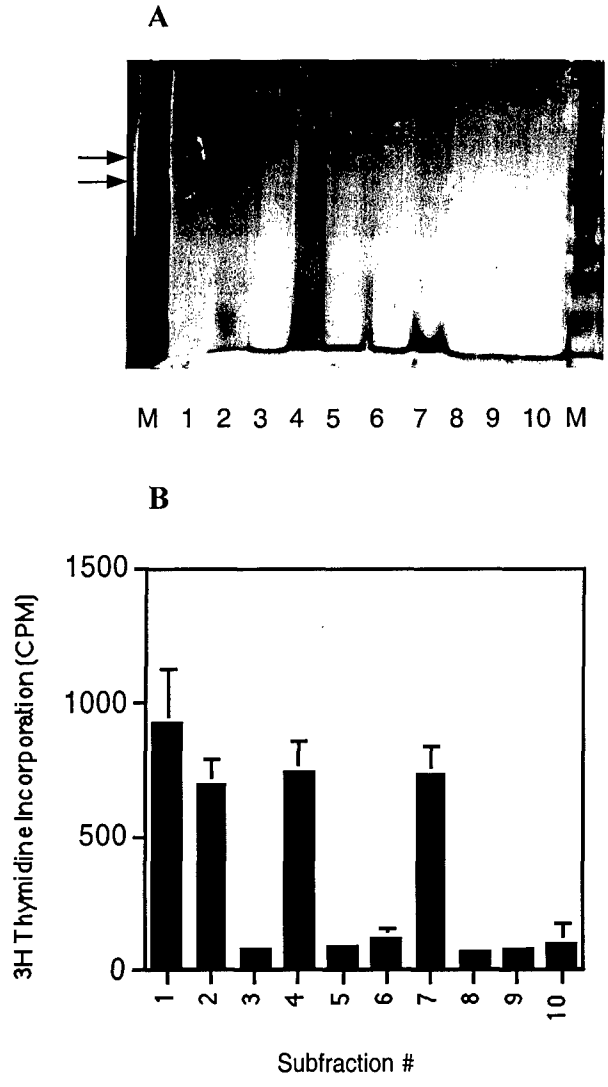
A
M 1 2 3 4 5 6 7 8 9 10 M
B
1500
1000
500
0
3H Thymidine Incorporation (CPM)
1 2 3 4 5 6 7 8 9 10
Subfraction #

FIGURE LEGENDS

Figure 1: Identification of 12 primed $CD8^+$ T cell cultures that recognized the original tumor, MS-A2, from which the tumor peptides were derived. The primed T cells were tested after the 4^{th} restimulation. E : T ratio was at 100:1. The Raji cell line (A3, B15, C7) was a control for alloreactivity.

Figure 2: $CD8^+$ T cells primed with peptide fraction # 32 from MS-A2 tumor recognized a shared tumor antigen on a lung tumor cell line, 201T-A2. $CD8^+$ T cells generated from priming to eluted peptides from fraction # 32 were used in a CTL assay after the 5^{th} restimulation.

Figure 3: $CD8^+$ T cells primed with pooled peptide fractions recognized the original tumor and were HLA Class I-restricted. A) $CD8^+$ T cells primed with peptide fractions #41-46 recognized the original tumor, MS-A2 and were blocked by the anti-MHC Class I antibody, W6/32. B) $CD8^+$ T cells primed with peptide fractions #61-65 recognized the original tumor (MS-A2), and not an HLA-matched tumor, Mel 624.

Figure 4: Identification of 12 primed $CD4^+$ T cell cultures that recognized the original tumor, MS, from which the proteins were obtained. The primed $CD4^+$ T cells were tested in a proliferation assay using macrophages loaded with UV-B induced apoptotic tumor (20 : 1 = T : macrophages) after the 2^{nd} restimulation.

Figure 5: $CD4^+$ T cell cultures primed with protein fractions recognize autologous macrophages loaded with tumor lysate. 2×10^6 autologous macrophages were loaded with ~ 1.75×10^8 cell

equivalents of tumor lysate for 2 hours and used as stimulators of primed $CD4^+$ T cell cultures in a proliferation assay. T cell cultures were pooled as indicated. T cells were added at a T cell : macrophage ratio of 10 : 1.

Figure 6: SDS PAGE and functional analysis of subfractions # 44.1 - # 44.10. Fraction #44 was subfractionated as described in *Materials & Methods*, and 10 subfractions were collected. A) 33% of each HPLC sub-fraction (# 44.1-# 44.10) was resolved on a 15% SDS-PAGE gel and silver stained. B) 33% of each sub-fraction was loaded onto 5 x 10^4 macrophages overnight and added to T cells primed to Fraction # 44 with a T : stimulator ratio of 1 : 1 for a 5-day proliferation assay. M, molecular weight markers.

REFERENCES

1. Brichard, V., Van Pel, A., Wolfel, T., Wolfel, C., De Plaen, E., Lethe, B., Coulie, P., and Boon, T. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas, J Exp Med. *178:* 489-95, 1993.
2. Coulie, P. G., Brichard, V., Van Pel, A., Wolfel, T., Schneider, J., Traversari, C., Mattei, S., De Plaen, E., Lurquin, C., Szikora, J. P., and et al. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas [see comments], J Exp Med. *180:* 35-42, 1994.
3. Hunt, D. F., Henderson, R. A., Shabanowitz, J., Sakaguchi, K., Michel, H., Sevilir, N., Cox, A. L., Appella, E., and Engelhard, V. H. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry [see comments], Science. *255:* 1261-3, 1992.
4. Hunt, D. F., Michel, H., Dickinson, T. A., Shabanowitz, J., Cox, A. L., Sakaguchi, K., Appella, E., Grey, H. M., and Sette, A. Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-Ad, Science. *256:* 1817-20, 1992.
5. Fisk, B., Blevins, T. L., Wharton, J. T., and Ioannides, C. G. Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines, J. Exp. Med. *181:* 2109-17, 1995.
6. Blake, J., Johnston, J. V., Hellstrom, K. E., Marquardt, H., and Chen, L. Use of combinatorial peptide libraries to construct functional mimics of tumor epitopes recognized by MHC class I-restricted cytolytic T lymphocytes, J Exp Med. *184:* 121-30, 1996.

7. Tureci, O., Sahin, U., and Pfreundschuh, M. Serological analysis of human tumor antigens: molecular definition and implications, Molecular Medicine Today. *3:* 342-9, 1997.

8. Wang, R. F. and Rosenberg, S. A. Human tumor antigens for cancer vaccine development, Immunol Rev. *170:* 85-100, 1999.

9. Traversari, C., van der Bruggen, P., Luescher, I. F., Lurquin, C., Chomez, P., Van Pel, A., De Plaen, E., Amar-Costesec, A., and Boon, T. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E, J Exp. Med. *176:* 1453-7, 1992.

10. van der Bruggen, P., Szikora, J. P., Boel, P., Wildmann, C., Somville, M., Sensi, M., and Boon, T. Autologous cytolytic T lymphocytes recognize a MAGE-1 nonapeptide on melanomas expressing HLA-Cw*1601, Eur. J. Immunol. *24:* 2134-40, 1994.

11. Gaugler, B., Van den Eynde, B., van der Bruggen, P., Romero, P., Gaforio, J. J., De Plaen, E., Lethe, B., Brasseur, F., and Boon, T. Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes, J. Exp. Med. *179:* 921-30, 1994.

12. van der Bruggen, P., Bastin, J., Gajewski, T., Coulie, P. G., Boel, P., De Smet, C., Traversari, C., Townsend, A., and Boon, T. A peptide encoded by human gene MAGE-3 and presented by HLA-A2 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE-3, Eur. J. Immunol. *24:* 3038-43, 1994.

13. Herman, J., van der Bruggen, P., Luescher, I. F., Mandruzzato, S., Romero, P., Thonnard, J., Fleischhauer, K., Boon, T., and Coulie, P. G. A peptide encoded by the human

MAGE3 gene and presented by HLA-B44 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE3, Immunogenetics. *43:* 377-83, 1996.

14. Boel, P., Wildmann, C., Sensi, M. L., Brasseur, R., Renauld, J. C., Coulie, P., Boon, T., and van der Bruggen, P. BAGE: a new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes, Immunity. *2:* 167-75, 1995.

15. Van den Eynde, B., Peeters, O., De Backer, O., Gaugler, B., Lucas, S., and Boon, T. A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanoma, J. Exp. Med. *182:* 689-98, 1995.

16. Disis, M. L. and Cheever, M. A. HER-2/neu oncogenic protein: issues in vaccine development, Crit Rev Immunol. *18:* 37-45, 1998.

17. Barratt-Boyes, S. M. Making the most of mucin: a novel target for tumor immunotherapy, Cancer Immunology, Immunotherapy. *43:* 142-51, 1996.

18. Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B., and Palucka, K. Immunobiology of dendritic cells [In Process Citation], Annu Rev Immunol. *18:* 767-811, 2000.

19. Macatonia, S. E., Taylor, P. M., Knight, S. C., and Askonas, B. A. Primary stimulation by dendritic cells induces antiviral proliferative and cytotoxic T cell responses in vitro, J. Exp. Med. *169:* 1255-64, 1989.

20. Macatonia, S. E., Patterson, S., and Knight, S. C. Primary proliferative and cytotoxic T-cell responses to HIV induced in vitro by human dendritic cells, Immunology. *74:* 399-406, 1991.

21. Mehta-Damani, A., Markowicz, S., and Engleman, E. G. Generation of antigen-specific CD8+ CTLs from naive precursors, J. Immunol. *153:* 996-1003, 1994.

22. Mehta-Damani, A., Markowicz, S., and Engleman, E. G. Generation of antigen-specific CD4+ T cell lines from naive precursors, Eur. J. Immunol. *25:* 1206-11, 1995.

23. Steinman, R. M. The dendritic cell system and its role in immunogenicity, Ann. Rev. Immunol. *9:* 271-96, 1991.

24. Celis, E., Tsai, V., Crimi, C., DeMars, R., Wentworth, P. A., Chesnut, R. W., Grey, H. M., Sette, A., and Serra, H. M. Induction of anti-tumor cytotoxic T lymphocytes in normal humans using primary cultures and synthetic peptide epitopes, Proc. Nat. Acad. Sci. *91:* 2105-9, 1994.

25. Tsai, V., Southwood, S., Sidney, J., Sakaguchi, K., Kawakami, Y., Appella, E., Sette, A., and Celis, E. Identification of subdominant CTL epitopes of the GP100 melanoma-associated tumor antigen by primary in vitro immunization with peptide-pulsed dendritic cells, J. Immunol. *158:* 1796-802, 1997.

26. Kawashima, I., Hudson, S. J., Tsai, V., Southwood, S., Takesako, K., Appella, E., Sette, A., and Celis, E. The multi-epitope approach for immunotherapy for cancer: identification of several CTL epitopes from various tumor-associated antigens expressed on solid epithelial tumors, Hum Immunol. *59:* 1-14, 1998.

27. Brossart, P., Stuhler, G., Flad, T., Stevanovic, S., Rammensee, H. G., Kanz, L., and Brugger, W. Her-2/neu-derived peptides are tumor-associated antigens expressed by human renal cell and colon carcinoma lines and are recognized by in vitro induced specific cytotoxic T lymphocytes, Cancer Res. *58:* 732-6, 1998.

28. Hiltbold, E. M., Ciborowski, P., and Finn, O. J. Naturally processed class II epitope from the tumor antigen MUC1 primes human CD4+ T cells, Cancer Res. *58:* 5066-70, 1998.

29. Hiltbold, E. M., Alter, M. D., Ciborowski, P., and Finn, O. J. Presentation of MUC1 tumor antigen by class I MHC and CTL function correlate with the glycosylation state of the protein taken Up by dendritic cells, Cell Immunol. *194:* 143-9, 1999.

30. Chaux, P., Vantomme, V., Stroobant, V., Thielemans, K., Corthals, J., Luiten, R., Eggermont, A. M., Boon, T., and van der Bruggen, P. Identification of MAGE-3 epitopes presented by HLA-DR molecules to CD4(+) T lymphocytes [see comments], J Exp Med. *189:* 767-78, 1999.

31. Finke, J. H., Zea, A. H., Stanley, J., Longo, D. L., Mizoguchi, H., Tubbs, R. R., Wiltrout, R. H., O'Shea, J. J., Kudoh, S., Klein, E., and et al. Loss of T-cell receptor zeta chain and p56lck in T-cells infiltrating human renal cell carcinoma, Cancer Res. *53:* 5613-6, 1993.

32. Finke, J., Ferrone, S., Frey, A., Mufson, A., and Ochoa, A. Where have all the T cells gone? Mechanisms of immune evasion by tumors, Immunol Today. *20:* 158-160, 1999.

33. Vega, M. A. and Strominger, J. L. Constitutive endocytosis of HLA class I antigens requires a specific portion of the intracytoplasmic tail that shares structural features with other endocytosed molecules, Proc. Nat. Acad. Sci. *86:* 2688-92, 1989.

34. Henderson, R. A., Michel, H., Sakaguchi, K., Shabanowitz, J., Appella, E., Hunt, D. F., and Engelhard, V. H. HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation [see comments], Science. *255:* 1264-6, 1992.

35. Kiessling, R., Kono, K., Petersson, M., and Wasserman, K. Immunosuppression in human tumor-host interaction: role of cytokines and alterations in signal-transducing molecules, Springer Seminars in Immunopathology. *18:* 227-42, 1996.

36. Ostrand-Rosenberg, S. Tumor immunotherapy: the tumor cell as an antigen-presenting cell, Cur. Opin. Immunol. *6:* 722-7, 1994.

37. Engelhard, V. H. Direct identification of tumor-associated peptide antigens, Springer Seminars in Immunopathology. *18:* 171-83, 1996.

38. Herr, W., Schneider, J., Lohse, A. W., Meyer zum Buschenfelde, K. H., and Wolfel, T. Detection and quantification of blood-derived CD8+ T lymphocytes secreting tumor necrosis factor alpha in response to HLA-A2.1-binding melanoma and viral peptide antigens, J Immunol Methods. *191:* 131-42, 1996.

Peer
10/29/2001

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND AND FORT DETRICK
810 SCHRIEDER STREET, SUITE 218
FORT DETRICK, MARYLAND 21702-5000

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 17 Oct 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for grants. Request the limited distribution statements for the Accession Document Numbers listed at enclosure be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Enclosure